Volume 215, number 1, 127–131 FEB 04655 May 1987

Patterns of gene expression during plasmacytoid differentiation of chronic lymphocytic leukaemia cells

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Received 24 February 1987

Chronic lymphocytic leukaemia (CLL) cells may be induced to undergo plasmacytoid differentiation in vitro in response to 12-*O*-tetradecanoyl phorbol acetate (TPA). We show here that plasmacytoid differentiation and the accompanying accumulation of Cμ immunoglobulin mRNA are preceded by a rapid transient increase in the expression of the proto-oncogenes, *c-myc* and *c-fos*. In terminally differentiated cells the level of *c-fos* mRNA returned to the original basal level whilst *c-myc* expression remained appreciably higher than in undifferentiated CLL cells. These data support a possible role for *c-fos* and *c-myc* in the programmed chain of events that occur during terminal differentiation of B-lymphocytes.

Chronic lymphocytic leukaemia; Differentiation; Oncogene expression

1. INTRODUCTION

B cell chronic lymphocytic leukaemia (CLL) represents a monoclonal expansion of immature B-lymphocytes that are arrested in their differentiation. When cultured in vitro in the presence of the phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) CLL cells can be induced to undergo terminal differentiation towards a plasma cell phenotype [1]. After 3 days post-induction, the cells display a loss of surface immunoglobulin and an increase in intracytoplasmic immunoglobulin and acquire ultrastructural features and phenotype of lymphoblasts [1,2]. This plasmacytoid differentiation thus provides a useful in vitro model in which to study mechanisms that co-ordinately regulate gene expression in the terminal differentiation of B-lymphocytes.

Studies on the expression of the nuclear proto-oncogenes, *c-myc* and *c-fos* during monocyte/macrophage differentiation have suggested a functional role for their gene products in the differentiation processes in this haemopoietic cell lineage [3–7]. Here, we have examined the expression of *c-myc* and *c-fos* during plasmacytoid differentiation of CLL cells and have compared this with the expression of immunoglobulin heavy chain (Cμ) mRNA. Our data reveal a unique pattern of expression which supports a role for these nuclear proto-oncogenes in the programmed chain of events occurring during terminal differentiation of B-lymphocytes.

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2. MATERIALS AND METHODS

CLL cells were isolated from the peripheral blood of three patients (CLL 1–3) by centrifugation on lymphoprep gradients (Nyegaard, Norway) and depleted of monocytes by adherence to plastic. The level of residual monocytes (determined by non-specific esterase staining) represented less than 1% of the total cell population. For induction of plasmacytoid differentiation, cells were cultured in RPMI medium supplemented with 10% foetal calf

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00145793/87/$3.50

serum and 16 nM TPA [1]. Morphological changes were apparent by 24 h and by 48 h most cells had acquired plasmacytoid features and became positive for intracytoplasmic immunoglobulin light chain.

At various time intervals, CLL cells were harvested and the total cellular RNA was extracted by the guanidinium isothiocyanate procedure [8]. The levels of *c-fos* and *c-myc* RNA were determined by an RNase protection assay using ^{32}P-labelled RNA probes (spec. act. 5×10^8 cpm/ng) generated from pSP64 plasmid constructs [9]. For *c-fos* a *Bam*HI + *Xmn*I fragment encompassing exon I (296 base pairs) and 192 base pairs of exon II cloned in pSP64 [10] was used as a template. A *c-myc* template was constructed by inserting a 340 base pair *Pst*I + *Hin*dIII fragment encompassing 200 base pairs of the 3′-end of *c-myc* exon II [11] and 120 base pairs of intervening sequence into pSP64. Synthesis of RNA probes and hybridisation with cellular RNA (performed overnight in a volume of 30 μl in sealed capillaries) were carried out as in [10]. After digestion with RNase A (40 μg/ml) and RNase T_1 (2 μg/ml) hybrids were denatured and electrophoresed on 5% polyacrylamide-8 M urea sequencing gels followed by autoradiography.

Immunoglobulin heavy chain mRNA was monitored by Northern blotting after electrophoresis of 5-μg samples of total cellular RNA on a 1% agarose-formamide gel. Hybridisation with an oligo-primed ^{32}P-labelled [12] 1.3 kbp probe encompassing the Cμ heavy chain locus [13] and post-hybridisation washes and autoradiography were performed as described [14].

3. RESULTS

Expression of the cellular proto-oncogene *c-fos* has not previously been studied in B CLL cells. Therefore, we initially examined the steady-state levels of *c-fos* mRNA in resting lymphocytes from two different CLL patients by using an RNase protection assay [9,10]. Fig.1A shows that a major fragment of 296 nucleotides corresponding to spliced *c-fos* exon I [10] was detected at a comparable level in both CLL RNA samples. We have also detected similar levels of *c-fos* in two other CLL cases not included in this study (not shown). When the assay was performed with RNA from the promyelocytic cell line HL60, before and after TPA-induced monocyte differentiation, an initial burst of *c-fos* expression was detected at 40 min

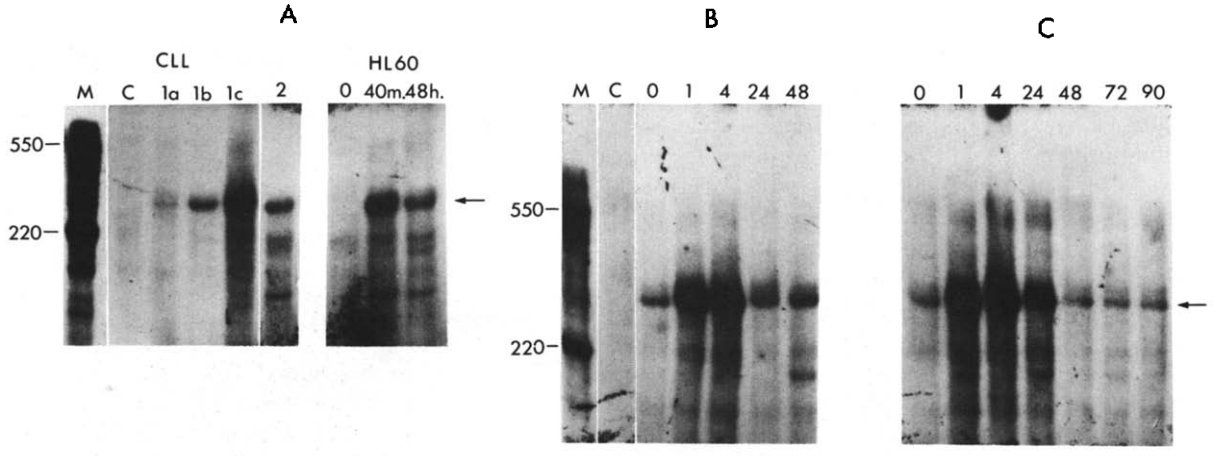

Fig.1. Levels of *c-fos* mRNA during in vitro differentiation of CLL cells. Various cell RNA samples were annealed overnight with *c-fos* probe, digested with RNase and electrophoresed on 5% polyacrylamide sequencing gels. Autoradiographs after a 4 day exposure are shown. (A) M, marker; C, control, tRNA (10 μg). 1a,1b,1c: 1, 4 and 20 μg RNA, respectively, from CLL 1. 2: 4 μg RNA from CLL 2. HL60 RNA (4 μg) at time 0, 40 min and 48 h (terminally differentiated) post-TPA treatment. (B) Time course RNA samples (4 μg) following TPA treatment of CLL 2 cells. M, marker; C, control, tRNA (10 μg). (C) Time course RNA samples following TPA treatment of CLL 3. Numbers indicate time (in h). B and C were performed in parallel and are taken from the same gel.

(fig.1A). In terminally differentiated HL60 cells (48 h) the level of *c-fos* RNA was reduced to a value still manifold higher than that in uninduced HL60 cells, consistent with previous work [6]. The level of *c-fos* RNA in resting CLL cells appeared to be comparable to that in terminally differentiated HL60 cells (fig.1A).

Fig.1B shows an analysis of *c-fos* mRNA during a time course of TPA-induced plasmacytoid differentiation of CLL 2 cells. By 1 h post-induction the *c-fos* level was elevated approx. 10-fold. This was sustained at 4 h but by 24 h *c-fos* mRNA was reduced to a level comparable to that in resting CLL cells. At 48 h post-induction when most cells were plasmocytoid, no further change in *c-fos* RNA was seen. Fig.1C shows a similar analysis for CLL 3 cells where a similar rapid, transient induction of *c-fos* was again seen. However, in this case *c-fos* RNA did not completely return to basal levels at 24 h. By 48 h *c-fos* did return to basal levels where it remained relatively constant up to 90 h post-induction. Since the experiments in fig.1B,C were performed in parallel, we attribute this minor difference in kinetics of disappearance of *c-fos* RNA to differences between cells from CLL 2 and 3.

The time course RNA samples from fig.1B,C were next examined for expression of the proto-oncogene *c-myc*. We chose an exon II probe in these studies to measure functional *c-myc* RNA (exon I is non-coding), since elongation of *c-myc* transcription may be blocked between exon I and intron I [15] although we have obtained identical data in initial experiments using an exon I probe (not shown). In RNA from resting CLL 2 cells *c-myc* transcripts were not detectable (time 0,

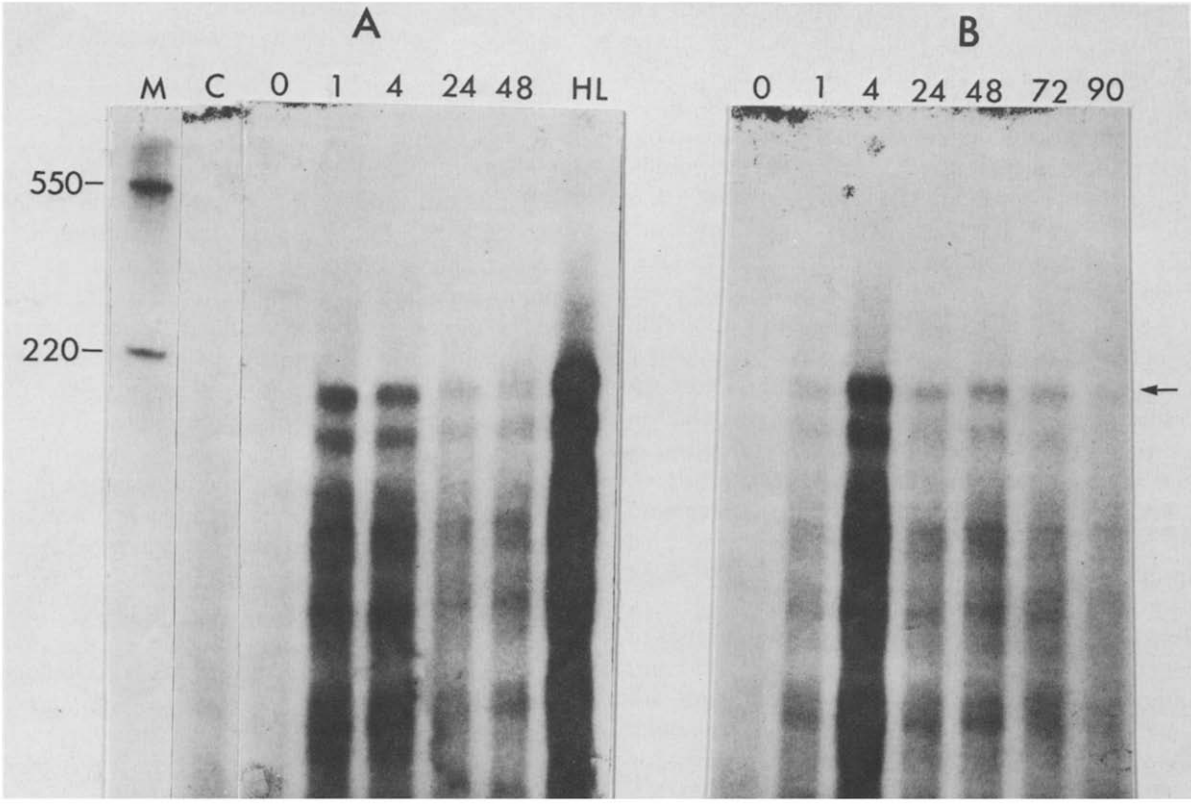

Fig.2. Levels of *c-myc* RNA during in vitro differentiation of CLL cells. Cell RNA samples (4 μg) from CLL 2 (A) and CLL 3 (B) or from uninduced HL60 cells (HL) were annealed overnight with *c-myc* probe then digested with RNase and electrophoresed on a 5% polyacrylamide sequencing gel. A and B are taken from the same gel. RNA samples and time points are exactly as described in fig.1B,C.

fig.2A) in agreement with earlier reports showing extremely low expression of this proto-oncogene in CLL [16]. At 1 h post-induction a burst in *c-myc* expression was seen in CLL 2 (fig.1A) which paralleled that of *c-fos*. The largest protected *c-myc* fragment was about 200 nucleotides long (marked with an arrow) consistent with the length of exon II sequences in the probe. However, in this and all other assays of *c-myc* RNA, a number of faster migrating fragments were reproducibly seen (fig.2) which could not be eliminated by modifying either the hybridisation or RNase digestion conditions in this assay. Similar spurious bands have been observed by other workers using RNase protection assays [17]. Although the origin of these fragments is not entirely clear they are present in roughly constant proportions in all samples examined and do not effect the quantitation of mRNA. In fig.2A we estimate the level of *c-myc* RNA in CLL2 at 1 h to be about one-fifth of that in promyelocytic HL60 cells which contain an amplified *c-myc* locus [18]. This level of *c-myc* RNA was sustained at 4 h but in contrast to *c-fos* the level was reduced only about 3–4-fold at 24 and 48 h post-induction and is thus appreciably higher than in resting CLL cells. An essentially similar result was obtained with RNA from CLL 3 cells (fig.2B) although as with *c-fos* there were minor differences in kinetics compared with CLL 2. Interestingly, *c-myc* RNA was noticeably lower at 1 h than at 4 h in CLL 3 (fig.2B) indicating that induction of *c-fos* probably precedes that of *c-myc* (cf. figs 1C,2B). In terminally differentiated CLL 3 cells *c-myc* RNA again remained higher than in resting CLL cells except for a small diminution at 90 h at which time cell viability is known to be lost.

Previously it has been shown that plasmacytoid differentiation of CLL cells is accompanied by a small increase in immunoglobulin heavy chain mRNA and a minor change in splicing pattern from the 2.7 kb mRNA (encoding membrane) to the 2.4 kb species (encoding secreted immunoglobulin) [19]. In order to compare the kinetics of this immunoglobulin mRNA accumulation with the changes in *c-fos* and *c-myc* expression a Northern blot analysis of time course RNA samples from CLL 3 was performed as shown in fig.3. Very little increase in immunoglobulin mRNA was seen over the first few hours post-induction. At 48 h there was a discernable increase

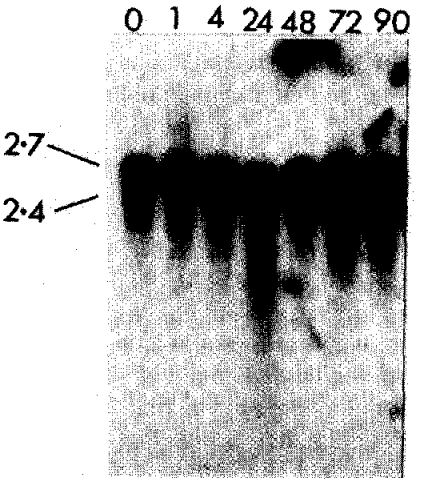

Fig.3. Levels of immunoglobulin heavy chain mRNA during in vitro differentiation of CLL cells. Northern blot analysis of RNA samples at times indicated (in h) is shown after hybridisation with immunoglobulin $C\mu$ gene probe. An autoradiograph is shown after a 4 day exposure. Sizes are given in kilobases. The signal obtained at 24 h appears slightly under-represented due to degradation which was apparent on the ethidium bromide-stained gel.

and at 72 h (essentially complete plasmacytoid differentiation) the level of immunoglobulin mRNA was about 2–3-fold higher than in resting CLL cells. This increase in accumulation of immunoglobulin RNA is comparable to that reported in [19] although we were unable to detect the minor change in relative abundance of mRNAs encoding membrane-bound and secreted immunoglobulin due to slight degradation of the RNA. Thus, in contrast to the rapid transient changes in *c-fos* and *c-myc* expression, the less dramatic change in immunoglobulin gene expression appears much later on in the plasmacytoid developmental programme.

4. DISCUSSION

In this report we have shown that TPA-induced plasmacytoid differentiation of CLL cells and the accompanying accumulation of immunoglobulin mRNA are preceded by a rapid, transient increase in expression of *c-fos* and *c-myc*. This pattern of oncogene expression has been observed in several cell types that are induced by a variety of agents to undergo differentiation or cell division [3–7,17] although this is the first report to our knowledge of

studies using a lymphocyte differentiation model. The functional role of the *c-fos* and *c-myc* gene products is currently highly speculative. The ability of these genes to disrupt cellular processes in tumourigenesis together with their aforementioned expression characteristics had led to the suggestion that at least one role may be to mediate pre-programmed nuclear functions in response to transduction of extracellular signals [7]. In the plasmacytoid differentiation of CLL cells the membrane receptor for TPA, protein kinase C, has previously been shown to be rapidly down-regulated (within minutes) in response to phorbol ester [20]. Thus, the kinetics of *c-fos* and *c-myc* expression which occurs over the first few hours but before onset of cellular differentiation changes would clearly be consistent with such a role.

The pattern of proto-oncogene expression during plasmacytoid differentiation contrasts markedly with that seen in monocyte/macrophage differentiation where there is a net increase in *c-fos* whilst *c-myc* is reduced in terminally differentiated cells [3–7]. In chemically induced terminal differentiation of mouse erythroleukaemia cells, *c-myc* shows an initial rapid decline followed by restoration to pretreatment levels after which *c-myc* RNA declines again [21]. It has been suggested that induction of differentiation leads to a change in the cell cycle regulation of *c-myc* expression [21]. Such a mechanism could account for the pattern of expression of this gene in plasmacytoid differentiation where the CLL cells traverse from G_0 to G_1 but cannot proceed to S phase (CLL cells are recalcitrant to induction of cell division and very little DNA synthesis occurs). In terminally differentiated macrophages the elevated expression of *c-fos* (also seen in normal marrow macrophages) has been interpreted as supporting a specific role of the *c-fos* gene product in terminally differentiated cells of this lineage [3,7]. The relatively high *c-fos* expression observed in resting CLL cells may reflect a similar functional role. The lack of net change on terminal differentiation would argue that CLL cells are blocked at a relatively late stage in B-lymphocyte maturation.

ACKNOWLEDGEMENTS

This work was supported in part by the Medical Research Council of Great Britain. We thank Professor A.V. Hoffbrand for support and encouragement.

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